A Novel Lung Slice System with Compromised Antioxidant Defenses

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In order to facilitate the study of oxidative stress in lung tissue, rat lung slices with impaired antioxidant defenses were prepared and used. Incubation of lung slices with the antineoplastic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (100 μM) in an amino acid-rich medium for 45 min produced a near-maximal (approximately 85%), irreversible inhibition of glutathione reductase, accompanied by only a modest (approximately 15%) decrease in pulmonary nonprotein sulfhydryls (NPSH) and no alteration in intracellular ATP, $NADP^+$, and NADPH levels. The amounts of NADP(H), ATP, and NPSH were stable over a 4-hr incubation period following the removal from BCNU. The viability of the system was further evaluated by measuring the rate of evolution of $^{14}CO_2$ from D-[^{14}C(U)]-glucose. The rates of evolution were almost identical in the compromised system when compared with control slices over a 4-hr time period. By using slices with compromised oxidative defenses, preliminary results have been obtained with paraquat, nitrofurantoin, and 2,3-dimethoxy-1,4-naphthoquinone.

Introduction

An important type of lung injury is produced by toxicants that induce an oxidative stress. Such a mechanism has been implicated in the pulmonary toxicity of the herbicide paraquat and the antimicrobial agent nitrofurantoin (*1*). Both compounds undergo a one-electron reduction catalyzed by microsomal NADPH-cytochrome P-450 reductase to form radical species. In the presence of molecular oxygen, the radical is oxidized with the generation of superoxide anion and reforms the parent molecule that can be rereduced forming a futile redox cycle. Compounds that redox cycle are characterized by a disproportionately greater consumption of oxygen, as compared with the concentration of the compound present (*2,3*). Active oxygen species so formed may induce toxicity by a number of different mechanisms including DNA-strand breakage, lipid peroxidation, and enzyme inactivation (*4*).

Despite extensive investigation, the mechanism of paraquat toxicity is not established, but the two major hypotheses propose an involvement of lipid peroxidation resulting from the production of active oxygen species and the depletion of the pyridine nucleotide, NADPH (*5–7*). These two mechanisms need not be mutually exclusive (*8*).

In order to combat the potential toxicity of active oxygen species, all cells possess antioxidant defenses (*2*). An important component of these is the glutathione reductase-glutathione peroxidase system. Glutathione reductase maintains intracellular glutathione, predominantly (> 95%) in the reduced form (GSH), by reducing oxidized glutathione (GSSG), formed by the peroxidase-catalyzed decomposition of hydrogen peroxide at the expense of NADPH (*9*).

The role of thiol status in paraquat toxicity is unclear. No thiol depletion is seen in rats dosed with paraquat (*10*), and sulfhydryl pretreatments do not ameliorate lethality (*11*). However, exogenous GSH protects alveolar Type II cells against loss of viability (*12*). Nitrofurantoin has been shown to cause a reduction in GSH levels in the isolated, perfused rabbit lung (*13*).

From clinical studies with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), it was observed that out of 20 erythrocytic enzyme activities investigated, only glutathione reductase was inactivated (*14*). Inactivation has been attributed to the carbamoylating activity of the 2-chloroethyl isocyanate moiety of BCNU (*15*).

BCNU has been employed in a variety of studies in isolated hepatocytes for the study of oxidative stress (*16,17*) and BCNU pretreatment potentiates hyperoxia-induced pulmonary fibrosis in mice *in vivo* (*18*). However, the maximal inhibition of glutathione reductase obtainable

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in vivo (~ 60%) is restricted by the dose-limiting toxicity of the compound.

The aim of the present study was to establish a model system with compromised oxidative defenses in order to study oxidative stress in the lung. Specifically, we wished to develop lung slices with a relatively specific inhibition of glutathione reductase.

Materials and Methods

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (London, UK) and were of the highest grade purity available. BCNU was obtained from Bristol-Myers Pharmaceuticals (Uxbridge, UK). Paraquat dichloride was a generous gift of ICI plc (Macclesfield, Cheshire, UK). 2,3-Dimethoxy-1,4-naphthoquinone was prepared as previously described (*19*). MEM amino acids were obtained from Gibco Ltd. (Paisley, Scotland). D-[^{14}C(U)]-Glucose was obtained from NEN Research Products (99% pure).

Male Wistar rats (160–250 g) were obtained from Olac Ltd. (UK). Lungs were perfused with Krebs Ringer phosphate (KRP) buffer with glucose, pH 7.4, and slices were prepared as previously described (*20*). Compromised slices were incubated with BCNU (100 μM) in KRP-enriched with amino acids. The amino acid-rich medium was prepared using MEM amino acid solution and appropriate additions of L-serine, L-cysteine, L-glycine, L-glutamine, and L-methionine (final concentration 0.2 mM), according to the specifications of Seglen (*21*). Slices were incubated for 45 min in a shaking water bath at 37°C. Slices were washed with KRP to remove BCNU and amino acids and used for biochemical measurements and incubations. Control slices were treated similarly except BCNU was omitted, and the slices were incubated in KRP in the absence of amino acids, as normal thiol levels were maintained in these slices under these conditions.

Lung slices were homogenized using a Polytron homogenizer for 30 sec. Samples for assay of NPSH and ATP were homogenized in 6.5% TCA/5 mM EDTA, whereas those for glutathione reductase were homogenized in potassium phosphate buffer, pH 7.4. Samples for NADPH assay were prepared in 0.1 M sodium hydroxide/0.1 M nicotinamide. For $NADP^+$, slices were homogenized in 0.5 M perchloric acid.

Glutathione reductase was measured using the spectrophotometric method of Carlberg and Mannervik (*22*). Nonprotein sulfhydryls were assayed using the method of Hissin and Hilf (*23*). This fluorometric assay is not specific for GSH. However, since GSH constitutes approximately 85% of lung thiols, NPSH measurements reflect GSH levels. ATP was assayed using a bioluminescence technique as previously described (*24*). Viability was evaluated using D-[^{14}C(U)]-glucose (1 μCi/center-well flask) (*20*). Samples were counted on a LKB-1216 Rackbeta liquid scintillation counter. Pyridine nucleotides were assayed using an established spectrophotometric method (*25*).

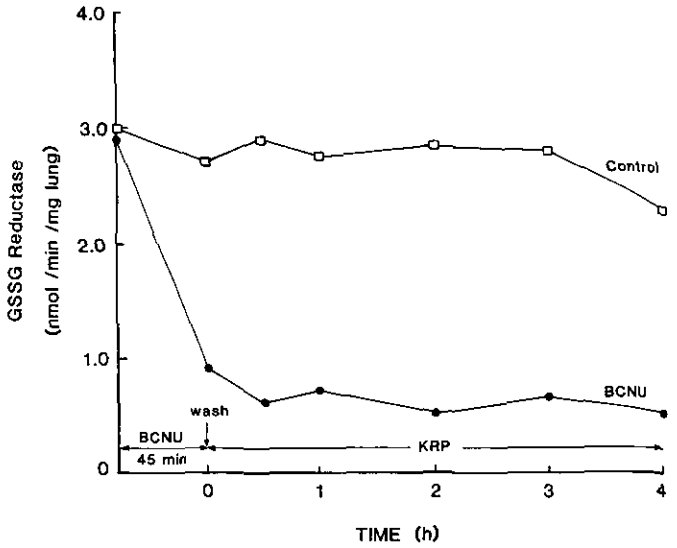


FIGURE 1. Effects of BCNU pretreatment on glutathione reductase activity. Lung slices were treated with DMSO (0.3%) (□—□) or with BCNU (100 μM in DMSO — a solubilizing agent for BCNU) and amino acids (●—●) for 45 min, then washed in Krebs-Ringer-phosphate (KRP). Results shown are from one experiment, typical of three.

Results

BCNU and Pulmonary Glutathione Reductase

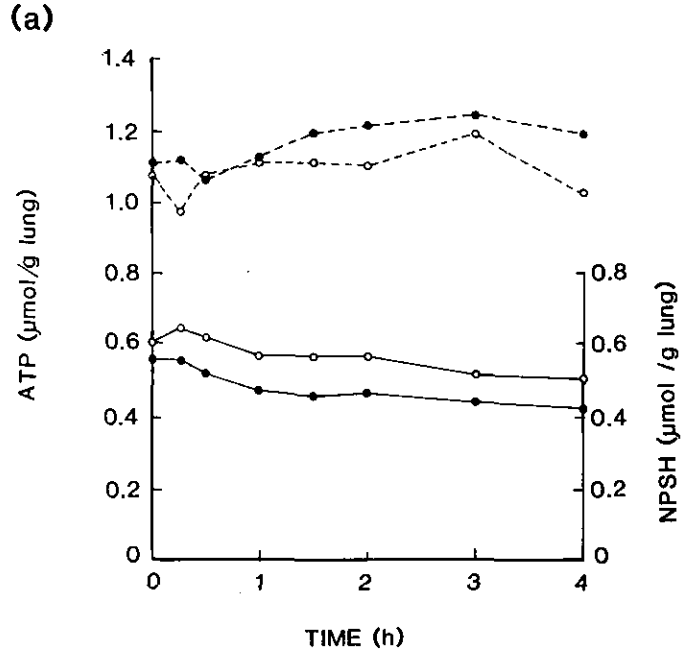
Isolation of lung slices in an amino acid-rich medium with BCNU (100 μM), as described in "Materials and Methods," resulted in a marked inhibition of glutathione reductase activity (80–85%), which remained constant over the 4-hr incubation period (Fig. 1).

BCNU and Other Biochemical Indices

In order to assess the effects of BCNU treatment on other biochemical markers related to oxidative stress and cell viability, NPSH, ATP, $NADP^+$, NADPH, and glucose oxidation were measured. A small but significant difference ($p < 0.05$), which was maintained throughout the 4-hr incubation, in NPSH levels, was observed following incubation with BCNU (Fig. 2*a*). Little or no differences were observed in the levels of ATP (Fig. 2*a*) and NADP(H) (Fig. 2*b*) following incubation with BCNU. Treatment of lung slices with BCNU had no effect on D-[^{14}C(U)]-glucose oxidation, in contrast to slices exposed to the mitochondrial inhibitor rotenone (Fig. 3).

Effects of Paraquat, Nitrofurantoin, and 2,3-Dimethoxy-1,4-Naphthoquinone in Control and Compromised Slices

Paraquat (100 and 500 μM) did not cause depletion of NPSH in control slices (Fig. 4). However, some depletion was seen in compromised lung slices (Fig. 4).



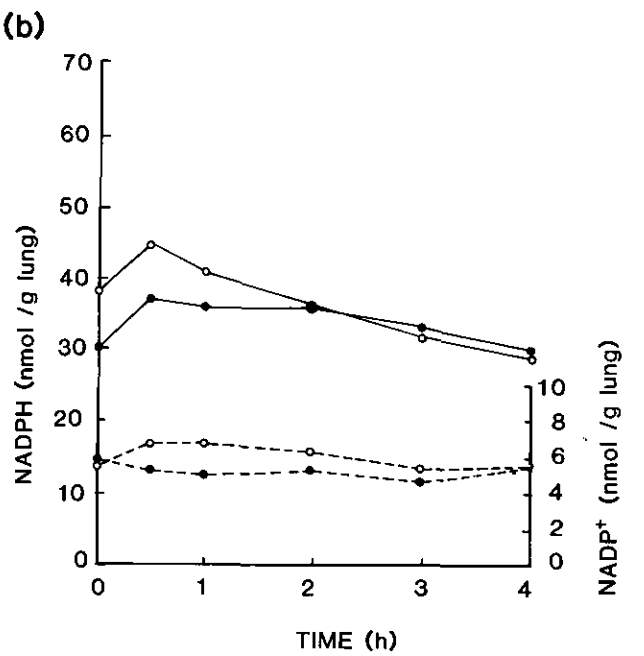


FIGURE 2. Effects of BCNU pretreatment on levels of NPSH, ATP, and NADP(H). Lung slices were incubated either as controls (○) or with BCNU (●). After a 45-min incubation and washing in KRP, the slices were incubated in DRP for various times and NPSH (—) and ATP (- - -) (*a*); NADPH (—) and $NADP^+$ (- - -) (*b*) were measured. The results represent the means of a minimum of seven determinations.

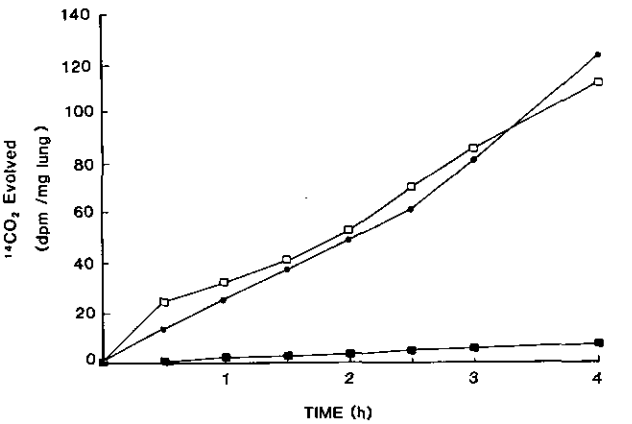


FIGURE 3. Glucose oxidation in control and BCNU-pretreated lung slices. Control (□—□) and compromised (●—●) slices were prepared as previously described. In addition, a set of control slices was treated with rotenone (100 μM) (■—■). Results are the means of three experiments. The basal amounts of $^{14}CO_2$ collected at zero time have been subtracted from the subsequent means.

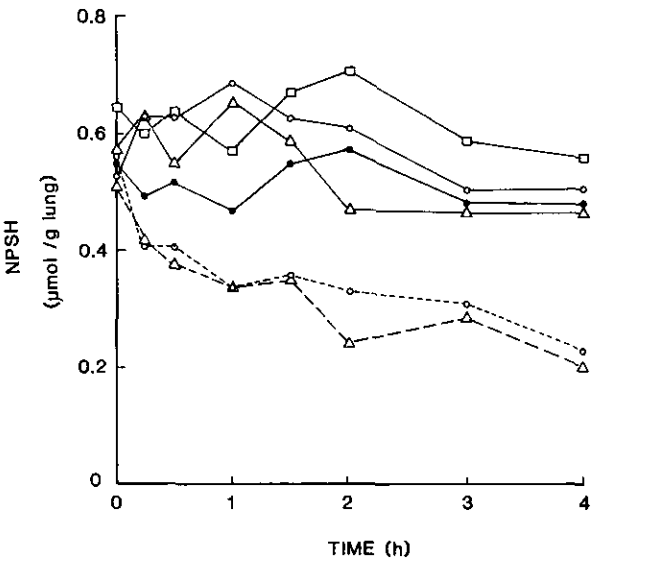


FIGURE 4. NPSH levels in lung slices treated with paraquat. Control slices were incubated in KRP in the absence (□—□) or the presence of 100 μM (○—○) or 500 μM (△—△)) paraquat. BCNU-pretreated slices were similarly incubated either in the absence (●—●) or the presence of 100 μM (○—○) or 500 △—△) paraquat, respectively. Results shown are from one experiment, typical of three.

Nitrofurantoin and 2,3-dimethoxy-1,4-naphthoquinone caused a concentration- and time-dependent depletion of NPSH in control lung slices. In preliminary studies, this depletion was potentiated in the compromised slices with nitrofurantoin (200 and 500 μM) and 2,3-dimethoxy-1,4-naphthoquinone (75 μM) (results not shown).

Discussion

BCNU has been employed in studies with isolated hepatocytes in order to study oxidative stress. Such pretreatment potentiates the toxicities of a number of compounds, including menadione, adriamycin, and diquat (*17,26*) that are proposed to be toxic by a mechanism involving oxidative stress. We wished to develop a lung slice model with compromised oxidative defenses, and therefore selected BCNU in an analogous manner to the earlier studies with hepatocytes.

Lung slices, incubated with BCNU, possessed a severely inhibited glutathione reductase (Fig. 1). Preincubation of slices with BCNU (100 μM) and amino acids for 45 min resulted in 80 to 85% inactivation, compared with glutathione reductase activities in control slices. This degree of inhibition is similar to that obtained with isolated hepatocytes (*16*) and greater than that obtained in lung tissue *in vivo* with the maximum nonlethal dose of BCNU (~ 60%) (*27*).

In our studies, inhibition of glutathione reductase with BCNU was time- and concentration-dependent (data not shown). Glutathione reductase activity observed in control slices was in good agreement with literature values (*27*). Inhibition of greater than 85% was achieved with either higher concentrations of BCNU or longer incubation times (results not shown), but these conditions resulted in a more marked loss of NPSH levels, compared to the small decrease observed (Fig. 2*a*).

Thus, we have described a simple, rapid (preparation time ~ 60 min) method for obtaining rat lung slices with a markedly inhibited glutathione reductase activity accompanied by a minimal decrease in NPSH, normal ATP (Fig. 2*a*), normal NADP(H) levels (Fig. 2*b*), and unchanged glucose oxidation (Fig. 3). The small NPSH depletion observed is unlikely to have a significant effect on the biochemical functions of the slices.

Preliminary results with compromised slices showed an apparent paraquat-induced NPSH depletion in contrast to control slices (Fig. 4). Similar results were observed in hepatocytes with the structurally related herbicide, diquat (*28*), and a role for GSH (and glutathione reductase) was implicated in diquat toxicity. These results suggested the possibility of a similar role for glutathione and reductase in response to paraquat in our studies. However, the magnitude of the NPSH change is small and its relevance to toxicity is still unclear.

The potentiation of thiol depletion with nitrofurantoin and 2,3-dimethoxy-1,4-naphthoquinone was observed in compromised slices (data not shown), and the significance of these effects is under investigation.

In summary, the development of a novel lung slice model with compromised oxidative defenses is described. The method has the advantages of speed and simplicity, and cell-to-cell interactions are maintained. We believe that the use of this model should increase our understanding of oxidative stress in the lung.

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